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Genetic, Temporal and Developmental Differences Between Melatonin Rhythm Generating Systems in the Teleost Fish Pineal Organ and Retina

J. Falcón,* Y. Gothilf,† S. L. Coon,‡ G. Boeuf* and D. C. Klein‡
*Laboratoire Arago, UMR 7628, CNRS and University Pierre and Marie Curie, Banyuls sur Mer, France.
†Department of Zoology, The George S. Wise Faculty of Life Sciences, Tel-Aviv University, Tel-Aviv, Israel.
‡NIH, Laboratory of Developmental Neurobiology, Bethesda, MD, USA.

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Abstract

Complete melatonin rhythm generating systems, including photodetector, circadian clock and melatonin synthesis machinery, are located within individual photoreceptor cells in two sites in Teleost fish: the pineal organ and retina. In both, light regulates daily variations in melatonin secretion by controlling the activity of arylalkylamine *N*-acetyltransferase (AANAT). However, in each species examined to date, marked differences exist between the two organs which may involve the genes encoding the photopigments, genes encoding AANAT, the times of day at which AANAT activity and melatonin production peak and the developmental schedule. We review the fish pineal and retinal melatonin rhythm generating systems and consider the evolutional pressures and other factors which led to these differences.

Melatonin is an important component of the vertebrate circadian system; it is made in two sites involved in phototransduction, the pineal gland and retina. Pineal-derived melatonin controls the daily rhythm in circulating melatonin and provides a hormonal signal of night time to the organism. This signal plays a role in the timing and control of a number of biological rhythms (1, 2). Retinal melatonin is thought to have a local paracrine function related to photic adaptation (3). In all vertebrates, melatonin production follows a circadian pattern controlled by a complex rhythm generating system. Such a system is typically composed of three basic elements, a photodetector, an endogenous clock and melatonin synthesizing machinery.

In Teleost fish, complete melatonin rhythm generating systems are located within individual photoreceptor cells in the pineal gland and retina (4). In most species, such as pike and zebrafish, this includes the three elements discussed above: a photodetector, circadian clock and melatonin synthesis machinery. As a result, when pineal glands from these species are placed in organ culture, melatonin production follows a circadian pattern (4, 5), which is a reflection of the clock. Light can modulate the circadian pattern of melatonin synthesis in two ways: (i) by resetting the clock and (ii) by blocking clock-stimulation of melatonin synthesis. By contrast to Teleost fish with 'clock-containing' melatonin rhythm generating systems, the clock is absent in trout and other salmonids. As a result, light is the only element controlling melatonin synthesis (4, 5).

This species-dependent difference is one reason why investigators are interested in how melatonin is regulated in fish. However, this is not the only reason. Differences also exist in the genes expressed in each tissue, which encode proteins with similar functions. However, the most puzzling differences between the Teleost fish pineal organ and retina are in the timing of the melatonin rhythm and responses to light. The Teleost fish pineal gland offers no surprises: it follows the same pattern seen in all vertebrates in that melatonin synthesis generally parallels the nocturnal increase in activity of the penultimate enzyme in melatonin synthesis, arylalkylamine *N*-acetyltransferase (AANAT; EC 2.3.1.87). AANAT controls the rate at which serotonin is converted to *N*-acetylserotonin (5), which earned this enzyme the title of 'the melatonin rhythm enzyme'. However, the retina is full of surprises. In trout, for example, retinal AANAT is elevated by lighting, not depressed, as is the case with pineal AANAT. In addition, in pike, the peak in retinal AANAT mRNA occurs in the light during late afternoon, in light, 6 h before pineal AANAT mRNA peaks in the middle of the night.

These and other differences will be reviewed here; in addition, some of the selective pressures and other factors responsible for

Correspondence to: Dr J. Falcón, Laboratoire Arago, UMR 7628, CNRS and University Pierre and Marie Curie, BP 44, F-66651 Banyuls sur Mer, France (e-mail: falcon@obs-banyuls.fr).

the evolution of different melatonin rhythm generating systems in the pineal organ and retina of Teleost fish are discussed.

Multiple copies of melatonin rhythm generating system genes: duplication of the fish genome

Proteins essential for generation of the rhythm in melatonin include several photopigment molecules which mediate effects of light and the enzymes involved in melatonin synthesis, such as AANAT. Analysis of the genes encoding some of these proteins reveals examples where two genes exist to encode the same protein or proteins with overlapping functions (6–15). The existence of duplicate genes in Teleost fish is not unusual and appears to be the result of a genome duplication early in the evolution between amphioxus and fish, following divergence from the main line leading to higher vertebrates. In the case of melatonin rhythm generating systems, this evolutionary event appears to have facilitated the evolution of somewhat different systems which, in some species, preferentially express one AANAT and one (or several) opsin-like molecules. In addition to this form of gene redundancy, there are differences in tissue expression of each gene. As described below, in some species, one family member can preferentially expressed in either the pineal gland or retina.

AANAT

In most vertebrates, only a single AANAT gene has been identified. However, two AANAT genes (AANAT1 and AANAT2) occur in Teleost fish (6, 9). In pike and trout, AANAT2 is expressed in the pineal organ, and AANAT1 is expressed in the retina and is more similar to the AANAT found in higher vertebrates. However, in zebrafish, AANAT2 is also dominant in the retina (16). The deduced amino acid sequences of the two AANATs in pike are 66% identical and 75% similar (6). The encoded proteins have distinct differences in their affinity for serotonin and in their relative affinity for indole-ethylamines versus phenyl-ethylamines; in addition, temperature-activity differences also exist (7, 8).

Photopigment

The growing list of photopigments found in fish includes rhodopsin, parapinopsin, vertebrate ancient opsin, exo-rhodopsin, UV opsin, and melanopsin (10–15). The relative distribution of each in the pineal organ compared to the retina is being characterized. Some, such as the zebrafish exo-rhodopsin, are preferentially expressed in the pineal organ, whereas canonical rhodopsin is expressed preferentially in the retina (11). Others, such as vertebrate ancient opsin, are expressed in the pineal gland, deep brain and nonvisual cells of the retina (12).

Other melatonin rhythm generating system proteins

In addition to opsins and AANATs, melatonin rhythm generating systems include a distinct set of proteins dedicated to phototransduction (e.g. transducin, arrestin, phosducin), circadian clock function (e.g. clock, period and cryptochrome) and melatonin synthesis (e.g. tryptophan hydroxylase and hydroxyindole-O-methyltransferase). It is not known whether any of these exist in duplicate and exhibit pineal- or retinal-specific expression. However, in Teleost fish, this issue will soon become more approachable, with the completion of the zebrafish and pufferfish genomes. Our preliminary analysis of the available uncharacterized zebrafish genome database has already provided evidence that two tryptophan hydroxylases genes may be present (Gothilf and Klein, unpublished data).

Temporal differences in the regulation of AANAT1 and AANAT2 activity

Clock-containing cells

The abundance of AANAT mRNA in pineal and retina cells of pike and zebrafish changes on a 24-h basis in animals maintained in a 12 : 12 h light/dark environment (6).

Temporal differences exist in the phasing of the rhythms

In pike, retinal AANAT1 mRNA peaks at dusk, 6 h before the mid-dark period peak in pineal AANAT2 mRNA. These daily rhythms are controlled by an endogenous circadian clock and therefore persist under constant lighting conditions of dim red light or constant light. Under these constant conditions, the phase-lag between the retinal and pineal rhythm in AANAT mRNAs is maintained, indicating the retinal and pineal clocks are differently regulated (6). The increase in AANAT mRNA is required for AANAT activity to increase (4, 5). The temporal differences may vary on an annual basis, as suggested from studies on the melatonin rhythm in sea bass (17) (Fig. 1).

Although the AANAT mRNA rhythms appear to persist independently of the environmental lighting cycle, this is not the case for rhythms in AANAT activity. In the pineal gland, light suppresses AANAT2 activity (18).

Non-clock containing cells of the trout

In the trout pineal gland, AANAT2 mRNA is continually elevated and not subject to either clock or photic regulation (19). Conversely, in the retina, AANAT1 mRNA is high at night and low during day under a LD cycle, but displays no variation under constant conditions (9). The only regulatory factor controlling AANAT1 mRNA abundance in trout, and probably other salmonids, is light (18).

Temporal differences also exist between the pineal gland and retina of the trout regarding the timing of the peak in AANAT activities and melatonin production. In the pineal gland, AANAT2 activity peaks in the dark, as is the case in most pineal glands (18). By contrast, in the retina, light has the opposite effect: it enhances AANAT1 activity and darkness suppresses it (Fig. 2). This is the first exception to the assumption that elevated melatonin is always associated with darkness. Accordingly, retinal melatonin levels are higher during day than during night, as observed in other Teleost fish species (17, 20–22).

Developmental differences

The pineal and parapineal organs, which make up the pineal complex, develop as two midline dorsal outgrowths of the diencephalic roof. The parapineal is transiently located rostral to the

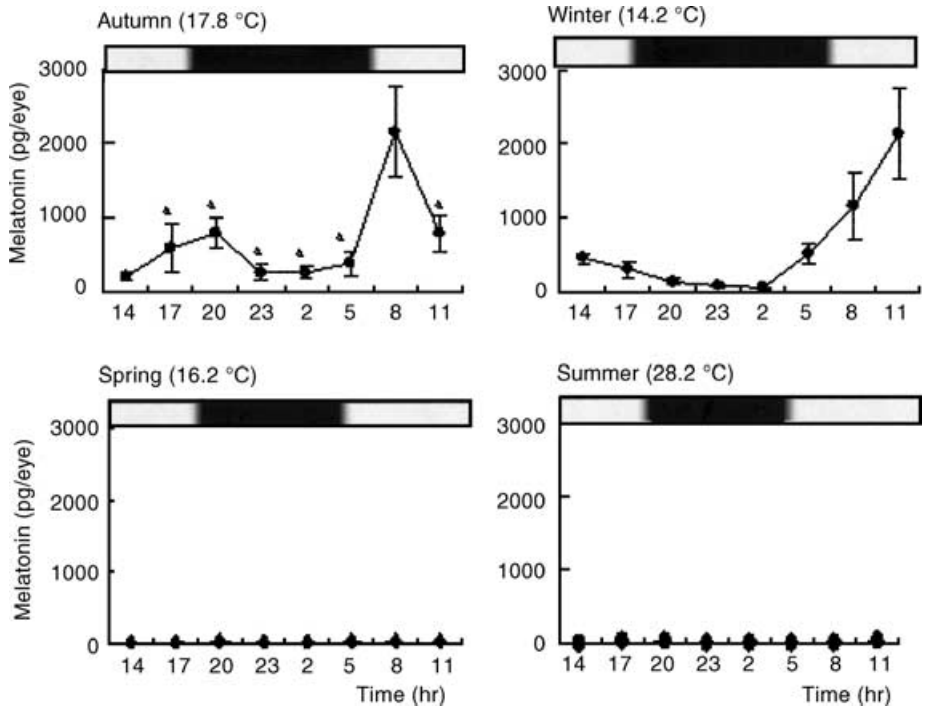


FIG. 1. Variations in retinal melatonin profiles in seabass (*Dicentrarchus labrax*) at different times of the year. Modified with permission (17).

pineal and later is situated unilaterally to the pineal. This pattern of development of the pineal complex was first described in a salmonid fish by Hill more than 100 years ago (23), was found in other fish species (24) and was recently re-confirmed using molecular markers (16, 25, 26).

Development of the pineal complex in Teleost fish occurs before that of the retina (16), and all elements of the melatonin generating system in the pineal organ are functional before the development of functional retinal photoreceptors. Studies in zebrafish indicate exorhodopsin and AANAT2 are expressed exclusively in the pineal primordium as early as 18 h and 22 h postfertilization, respectively (Fig. 3). A clock-controlled rhythmic expression of zfAANAT2 mRNA levels in the pineal complex begins at 2 days postfertilization (Fig. 3). This rhythm and the rhythm of melatonin production are established by the photic signal on the second day after fertilization (i.e. 24–36 h postfertilization) (16, 27) (Fig. 3).

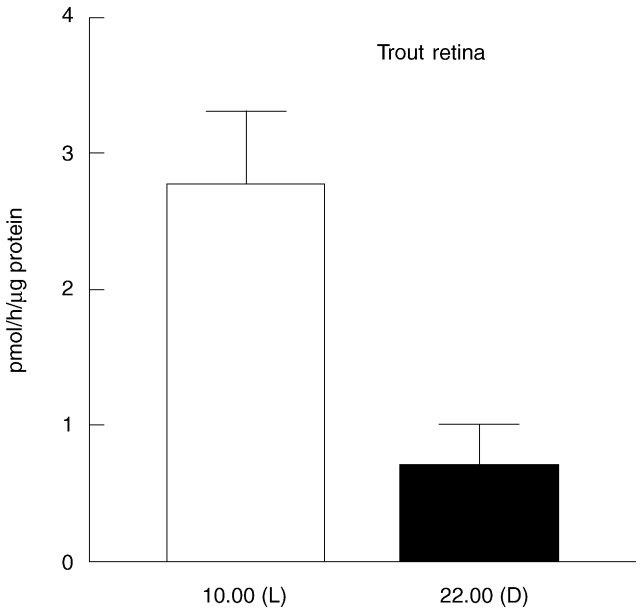


FIG. 2. Arylalkylamine *N*-acetyltransferase activity during a 24-h cycle. Trout were adapted for 24 h to a $11L_{(07.30-18.30\,h)}$/13D cycle, and then killed at the times indicated. Each plot represents the mean ± SEM of five individuals. $F_{LD} = 2.83$, $P < 0.046$ (ANOVA).

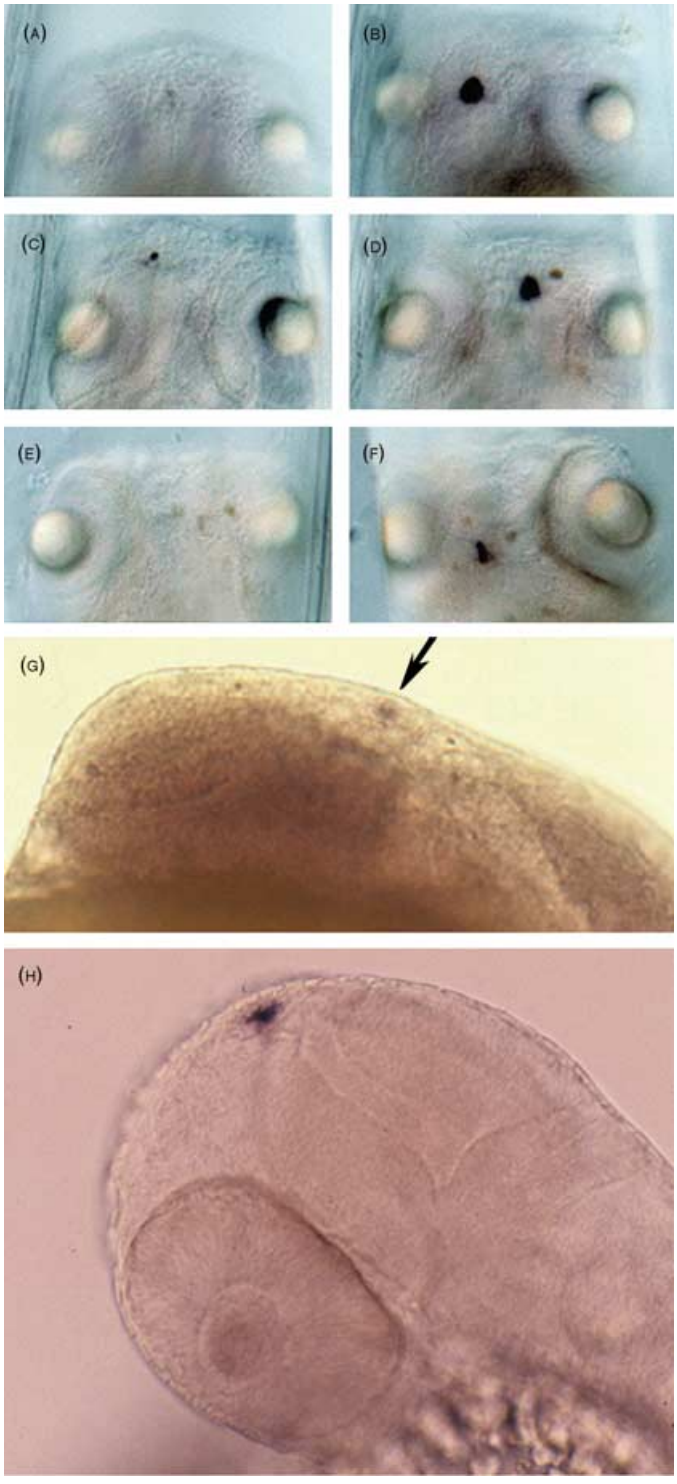
Retinal photoreceptors develop later; however, they seem to possess elements necessary for melatonin production. For example, in zebrafish, the first differentiating retinal photoreceptors can be found in the ventral part of the eye in the ventral patch (28). These cells express opsins and AANAT2 mRNA. As the retina continues to develop, the AANAT2 mRNA signal spreads dorsally throughout the retina until it covers the entire retina.

The presence of a photoreceptive, clock-containing and melatonin-producing pineal gland, before the development of functional retinal photoreceptors, reflects the autonomous nature of the Teleost fish pineal and suggests that prediction of the environmental light cycle at this stage is of selective advantage, and that pineal melatonin plays a role in this process (29). For example, the timing of hatching, which is obviously an important factor for survival, is affected by environmental light. By contrast, retinal melatonin, having a paracrine role is not needed for such functions.

Factors leading to differences in the fish pineal and retinal melatonin rhythm generating systems

It is clear that differences exist in the melatonin rhythm generating system in the pineal organ and retina of Teleost fish. It is reasonable to consider that the major factor driving the evolution of these differences is the different functions of melatonin derived from

each tissue. In the case of the pineal gland, melatonin is synthesized and released into the circulation, where it provides a reliable hormonal indicator of the day/night cycle (i.e. high at night and low during the day). This signal appears to have been highly conserved during the course of evolution. Although the signal is clearly somewhat plastic, in essentially all cases, circulating melatonin is high at night, which is a constant feature of vertebrate physiology (4, 5).



The situation in the retina is less clear. Although melatonin synthesis in the retina of some higher vertebrates appears to be enhanced at night, similar to the pineal gland, this is not 'the rule' in fish, where melatonin synthesis in some cases is high during the day or late in the afternoon (17, 20–22). These pineal versus retinal differences in the temporal pattern of melatonin production are probably linked to a paracrine function of melatonin in photodetection. Although this role of melatonin is currently poorly understood, this situation should change as more is learned about the temporal pattern of melatonin production and these advances are integrated into knowledge of other daily rhythmic functions. The eye is one of the most adaptable and varied structures in vertebrates, reflecting the critical role it plays in the life style and survival of an animal. Accordingly, it is not surprising that such adaptation and variation is also seen in retinal melatonin production.

Differences have also been observed in the 'tightness' of the association of the retinal rhythm with the environmental lighting schedule within one species. For example, in the sea bass, the rhythm in pineal melatonin and circulating melatonin is always linked to the environment; both are high at night. However, the association of the retinal rhythm in melatonin with environmental lighting varies on a seasonal basis (17).

There are two other factors to consider when discussing the existence of marked pineal/retinal differences. One is duplication of the fish genome, which generated the opportunity for duplicate genes to exist and to evolve independently. The presence of different opsins, AANATs and perhaps other proteins involved in phototransduction, circadian clock function and melatonin production, enhances the opportunity to fine tune photodetection so that it optimally functions in the pineal organ for hormonal purposes and in the retina for paracrine purposes. Different life styles introduce different pressures on these systems.

The second factor is the large number of progeny in each reproductive cycle of fish, which allows for rapid adaptive changes to occur (i.e. for a single mutation in the germ line to be immediately amplified and passed on to a large population).

The remarkable differences seen in Teleost fish (both between species and between the pineal gland and retina) appear to reflect the interaction of three factors: adaptation, gene duplication and rapid introduction of germ line mutations to a population. In the case of the fish retina, which exhibits remarkable differences and 'exceptions to the rule', this seems to reflect the high degree of adaptation characteristic of the vertebrate retina (i.e. a fine tuning), which enhances the ability of the animal to detect images, as required for survival. The fish retina may provide investigators with an excellent opportunity to learn more about the role of melatonin

FIG. 3. Exclusive expression of AANAT2 (A–F) and exorhodopsin (G, H) mRNA in the pineal primordium and gland of zebrafish embryos. The collected embryos where processed for whole mount *in situ* hybridization using DIG-labelled antisense RNA probes. Zebrafish exorhodopsin and AANAT2 probes were prepared as described elsewhere (11, 16), respectively. (A–F) AANAT2. Embryos were raised under a 14 : 10 h light/dark cycle and collected at the indicated times. The dorsal views of the head regions are shown. (A) Day-time expression in 56-h postfertilization embryo, CT = 8. (B) Night-time expression in 65-h postfertilization embryo, CT = 17. (C) Day-time expression in 79-h postfertilization embryo, CT = 7. (D) Night-time expression in 91-h postfertilization embryo, CT = 19. (E) Day-time expression in 104-h postfertilization embryo, CT = 6. (F) Night-time expression in 116-h postfertilization embryo, CT = 20. (G, H) Exorhodopsin is specifically expressed in the pineal primordium at 18 h (G) and 56 h (H) postfertilization.

in this process and, as a result, provide a better understanding of the function of AANAT and melatonin in the vertebrate retina.

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